



## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Chicheportiche, et al.

Art Unit : 1635

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Examiner : R. A. Schnizer

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Title : A TUMOR NECROSIS FACTOR RELATED LIGAND

Commissioner for Patents

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SECOND DECLARATION OF JEFFREY BROWNING, PH.D.UNDER 37 C.F.R. § 1.132

I, Jeffrey Browning, Ph.D., hereby declare and state as follows:

1. I am one of the named co-inventors of the above-identified patent application. I currently hold the position of Distinguished Investigator at Biogen Idec, one of the co-assignees of this application. My resume and qualifications are more fully described in ¶ 1 of the (first) Declaration of Jeffrey Browning, Ph.D., executed on February 6, 2004 and mailed to the Office on February 25, 2004.

2. I understand that during the telephone conference dated May 25, 2005 the Examiner inquired about the protein encoded by the construct described at page 34 of the specification. In the Interview Summary dated May 27, 2005, the Examiner requested that I clarify "what fragment of the human protein had actually been expressed, (i.e., what fragment is encoded by the PpuMI-NotI construct discussed at page 34 of the specification)."

3. The specification states, within the paragraph bridging pages 34-35, that:

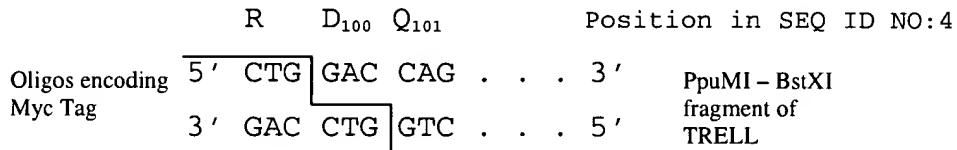
The following DNA fragments were isolated, a Not1/blunt fragment encoding the VCAM leader and a pair of oligonucleotides encoding the myc tag (5' blunt, 3' PpuM1 site) which have been described, a 0.45 kb PpuM1/BstX1 fragment of TRELL and a 0.65 BstX1/Not1 fragment of TRELL. The four fragments were ligated into a Not1/phosphatased pBluescript vector. The Not1 insert from this vector was transferred into the pFastBac1 vector (GibcoBRL) and used to generate recombinant baculovirus. Soluble TRELL was prepared by infecting HiFive™ insect cells at a MOI of 10 and the medium was harvested after 2 days.

This construct is also described in Chicheportiche et al., (1997) *J. Biol. Chem.* 272:32401-32410 (Exhibit A), particularly at page 32402, left column bottom at the paragraph titled, "Expression of Recombinant hTWEAK protein." TWEAK is the current name for the protein described as TRELL in the application.

4. A general schematic of the NotI insert in the construct is indicated below:



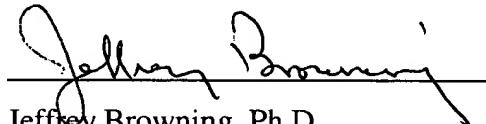
A detailed view of the sequence at the PpuMI site (an enzyme which cleaves leaving a 5' overhang by cleaving RG<sup>5</sup>GWCCY) is shown below.



As seen above, the human TRELL coding sequence (right side) was joined to annealed oligonucleotides encoding the Myc Tag (left side) using the overhangs from a PpuMI restriction site (nucleotides 295-301 of SEQ ID NO:3) in the TRELL coding sequence. The codon within the overhang encodes amino acid 100 of SEQ ID NO:4. This amino acid is aspartic acid (D). Ligation of the PpuMI-BstXI fragment to the BstXI-NotI fragment produced a sequence that encodes amino acids 100 to 284, the C-terminus of human TRELL. Accordingly, the protein encoded by the construct described on page 34 of the specification is a fusion of the VCAM leader, the myc epitope tag and a fragment of human TRELL that corresponds to amino acids 100 to 284 of SEQ ID NO:4.

5. The application also discloses at least a functional part of murine TRELL. An alignment of a murine TRELL amino acid sequence to the human TRELL amino acid sequence is shown in FIG. 1 of the application. The alignment is complete within the region defined by the fragment of human TRELL that was expressed by the construct described on page 34 of the specification. The corresponding fragment of murine TRELL is easily identifiable from the alignment.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



Jeffrey Browning, Ph.D.  
Cambridge, MA  
Date: 6-23-05



## Exhibit A

# TWEAK, a New Secreted Ligand in the Tumor Necrosis Factor Family That Weakly Induces Apoptosis\*

(Received for publication, July 10, 1997, and in revised form, September 22, 1997)

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The members of the tumor necrosis factor (TNF) family play pivotal roles in the regulation of the immune system. Here we describe a new ligand in this family, designated TWEAK. The mouse and human versions of this protein are unusually conserved with 93% amino acid identity in the receptor binding domain. The protein was efficiently secreted from cells indicating that, like TNF, TWEAK may have the long range effects of a secreted cytokine. TWEAK transcripts were abundant and found in many tissues, suggesting that TWEAK and TRAIL belong to a new group of widely expressed ligands. Like many members of the TNF family, TWEAK was able to induce interleukin-8 synthesis in a number of cell lines. The human adenocarcinoma cell line, HT29, underwent apoptosis in the presence of both TWEAK and interferon- $\gamma$ . Thus, TWEAK resembles many other TNF ligands in the capacity to induce cell death; however, the fact that TWEAK-sensitive cells are relatively rare suggests that TWEAK along with lymphotoxins  $\alpha/\beta$  and possibly CD30L trigger death via a weaker, non-death domain-dependent mechanism.

Cytokines of the TNF<sup>1</sup> family are mediators of host defense and immune regulation. Members of this family act either locally through direct cell-to-cell contact or as secreted proteins capable of diffusing to more distant targets. These proteins are synthesized as type II membrane proteins with the extracellular C-terminal region mediating binding to the receptors of the TNF receptor (TNF-R) family (1). The TNF family of ligands and receptors comprises at least 14 unique signaling pathways including TNF, lymphotoxins (LT), Fas, CD27, CD30, CD40, 4-1BB, OX-40, TRAMP (also DR3, WSL-1, Apo-3), CAR-1, TRAIL, GITR, HVEM, osteoprotegerin, and NGF (2–12). Excluding NGF, each of these signaling pathways is likely to be

involved in critical functions related to both the function and development of the immune system. For example, TNF acts primarily as an inflammatory cytokine coordinating host defenses in response to aggression by pathogens by activating a wide range of immunological and non-immunological mechanisms (13). The LT system is involved in the development of the peripheral lymphoid organs and the organization of splenic architecture (14, 15). CD40 is a key element in the regulation of the immunoglobulin response (16), and Fas signaling has been implicated in the mechanisms controlling peripheral tolerance and thymic selection (17, 18). Other members such as OX-40, 4-1BB, CD27, and CD30 are also involved in the control of various aspects of the immune system (19–22).

Some of these receptors upon activation can directly trigger the apoptotic death of many transformed cells, e.g. TNF-R55, Fas, TRAIL-R, and TRAMP (23). Clearly, Fas and possibly TNF-R55 and CD30 activation can induce cell death in non-transformed lymphocytes, which may play an immunoregulatory function (22–25). In general, death is triggered following the aggregation of death domains that reside on the cytoplasmic side of the TNF receptors. The death domain orchestrates the assembly of various signal transduction components that result in the activation of the caspase cascade (23). Some receptors lack canonical death domains, e.g. LT $\beta$  receptor and CD30 (26, 27), yet can induce cell death, albeit more weakly. It is likely that these receptors function primarily to induce cell differentiation *in vivo* and the death is an aberrant consequence in some transformed cell lines, although this picture is unclear, as studies on the CD30 null mouse suggest a role in death during negative selection in the thymus (22). Conversely, signaling through other pathways such as CD40 is required to maintain cell survival.

In this paper, we describe the mouse and human versions of a new member of this family, which we have called TWEAK. Like TNF, TWEAK is readily secreted from cells. Furthermore, we have prepared a recombinant soluble form of the molecule and used it to show that TWEAK signaling can induce cell death in an adenocarcinoma cell line.

## EXPERIMENTAL PROCEDURES

**Cells and Reagents**—All cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and grown in the recommended medium except for WEHI 164 clone 13, which was obtained from Dr. Kawashima (Geneva Biomedical Research Institute, Geneva, Switzerland). The HT29 subclone (HT29-14) and II-23 T cell hybridoma have been described previously (26, 28), and the TNF-sensitive ME180 subclone was obtained from Dr. Carl Ware. Recombinant FLAG-labeled hTRAIL was a generous gift from Dr. J. Tschopp (3), and anti-FLAG M2 monoclonal antibody was purchased from Eastman Kodak Corp. Anti-Fas CH11 was purchased from Kamiya Biomedical Co. (Seattle, WA), and LT $\alpha 1/\beta 2$  was prepared as described previously (29).

**Mouse TWEAK Cloning**—The antisense oligonucleotide primer 5'-GTTCCAGGCCAGCCTGGG-3' from the mouse erythropoietin se-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF030099 and AF030100.

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<sup>1</sup> The abbreviations used are: TNF, tumor necrosis factor; IL, interleukin; TNF-R, TNF receptor; NGF, nerve growth factor; RACE, rapid amplification of cDNA ends; LT, lymphotoxin; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; IFN, interferon; EBNA, Epstein-Barr virus nuclear antigen; DAPI, 4',6-diamidino-2-phenylindole.

quence was used in a 5'-RACE protocol following the recommendation of the manufacturer (5'-RACE system from Life Technologies, Inc.) in association with the Life Technologies-designed anchor primer. Template cDNA was made from RNA from 1-h adherent peritoneal macrophage RNA. After a 5-min denaturation at 94 °C, the cycling conditions were as follows: 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 3 min at 72 °C followed by a terminal extension step at 72 °C. Analysis of the PCR products on an agarose gel revealed two amplified fragments of 650 and 500 bp. The two fragments were excised from the gel, inserted in pBS-T vectors and sequenced. Northern hybridizations with <sup>32</sup>P-labeled random-primed fragments indicated that the 500-bp fragment hybridizing to a 1.4-kb RNA in macrophages. To determine the orientation of the cDNA, <sup>32</sup>P-labeled riboprobes in both directions were used in Northern hybridization. From the determined orientations and sequences, we derived two internal primers for the 1.4-kb mRNA: 5'-TCAGGTGCACCTTGATGAGG-3' and 5'-CTGTAGCTCCTCTGAG-3', which were used in 3'- and 5'-RACE PCR, respectively. The 3'-RACE experiment revealed a 750-bp fragment, which was inserted in pBS-T vector and sequenced. It corresponded to the 3' end of the 1.4-kb RNA since the sequence possessed a poly(A) addition signal just prior to the poly(A) tract. The 5'-RACE did not reveal any band. The CLONTECH Marathon cDNA amplification kit was used to prepare a cDNA library from 1-h adherent macrophages. PCR used a 1040-bp TWEAK PCR fragment and the universal primer from the kit. The 1040-bp TWEAK PCR fragment was isolated using sense and antisense oligonucleotide primers from the determined cDNA sequence (5'-AGCAGGAGCCTCTCAGGAG-3' and 5'-GATCCAGGGAGGAGCTTGCC-3'). This method resulted in the isolation of a fragment 60 bp longer on the 5' end than the original 1040-bp fragment.

**Human TWEAK Cloning**—A search of the EST data base revealed 1 human clone that was clearly homologous to the murine sequence. The clone 154742 (GenBank™ accession no R55379) has a 345-bp sequence 89% homologous to the murine cDNA. Two primers derived from the EST (5'-CCCTGCGCTGCCTGGAGGAA-3' and 5'-AGACCAGGGCC-CCTCAGTGA-3') were used in RT-PCR reactions to screen different tissues and libraries for the presence of hTWEAK transcripts. Products of the correct size were obtained from liver, spleen, lymph node, THP-1, and tonsil, but not from U937 mRNA. The 201-bp product was cloned and used to screen a λgt10 human tonsil cDNA library. One million plaque-forming units were plated at 10<sup>5</sup> plaque-forming units/plate. Duplicate lifts were made onto 20 × 20-cm nitrocellulose filters and hybridized with a probe prepared by random priming. The filters were hybridized overnight at 65 °C in plaque screen buffer (50 mM Tris, pH 7.5, 1 M NaCl, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidone, and 0.2% Ficoll) containing 10% dextran sulfate, 100 µg/ml tRNA, and 6 × 10<sup>6</sup> cpm/ml of probe. They were washed twice with plaque screen buffer and twice with 2 × SSC, 0.1% SDS at 65 °C. λ miniprep DNAs were prepared from positive colonies, and the clones with the largest inserts were selected for large scale DNA purification and DNA sequencing. The inserts were subcloned into the *NotI* site of pBlueScript SK+.

**RNA Analysis**—Either a 0.45-kb *PpuMI/BstXI* or a 1.25-kb *NarI/NotI* fragment of the hTWEAK cDNA was labeled by random priming and used to probe human adult tissue and cell Northern blots purchased from CLONTECH. Blots were probed as per the manufacturer's instructions and given a final wash at 0.1 × SSC with 0.1% SDS at 50 °C.

**Chromosomal Assignment**—A panel of DNA from monochromosomal cell hybrids (HGMP Resource Center, Hinxton, Cambridge, UK) was used to amplify a 329-bp fragment with primers chosen in 3'-untranslated region that are not homologous to the murine sequence (5'-AGTCGTCCCAGGCTGCCGGCT-3' and 5'-CCTGAAGTGCGGTCTGGAA-3'). Amplification was done for 40 cycles, 30 s at 94 °C, 90 s at 65 °C, and 90 s at 72 °C. Additional mapping was carried out using the same PCR primers on the Genebridge 4 Radiation Hybrid DNA panel (HGMP Resource Center), and the results were analyzed using the RHMAPPER program at the Whitehead Institute.

**Expression of Recombinant hTWEAK Protein**—A soluble expression construct combining the VCAM leader sequence, the Myc peptide tag, and the extracellular domain of hTWEAK was prepared similar to that described for LTβ (29). The following DNA fragments were isolated or synthesized, a *NotI*/blunt fragment encoding the VCAM leader, a pair of oligonucleotides encoding the Myc tag (5' blunt, 3' *PpuMI* site; 5'-GAACAGAAAATCTCATCTCTGAAGAAGACCTG and 5'-GTCCAGGT-CTTCTCAGAGATGAGTTCTGTTC), a 0.45-kb *PpuMI/BstXI* fragment of TWEAK, and a 0.65-kb *BstXI/NotI* fragment of TWEAK. The four fragments were ligated into a *NotI*/phosphatased pBlueScript vector. The *NotI* insert from this vector was transferred into the pFastBac1 vector (Life Technologies, Inc.) and used to generate recombinant bacu-

lovirus. Soluble TWEAK was prepared by infecting HiFive™ insect cells at a multiplicity of infection of 10, and the medium was harvested after 2 days. The following items were added to the media: HEPES buffer to a final concentration of 25 mM, pH 7.4, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, Calbiochem), and 1 µg/ml pepstatin. The media was filtered and concentrated 10-fold by ultrafiltration over a Amicon 10-kDa cut-off filter. The concentrated medium was loaded directly onto a Fast S column, washed with 25 mM HEPES buffer, pH 7.4, with 0.4 M NaCl, and eluted with 0.7 M NaCl in the same buffer. N-terminal amino acid sequencing was performed on blot transfers following SDS-PAGE.

**Analysis of TWEAK Secretion**—Vectors for EBNA-based expression were constructed using the vector CH269, which is a modified version of the pCEP4 (Invitrogen) from which the EBNA gene was removed. A 0.71-kb fragment of hTNF in the pFastBac vector was provided by Dr. P. Pesavento and A. Goldfeld (Dana Farber Cancer Institute). The *SnaBI/XbaI* insert was ligated into the *PvuII/XbaI* site of CH269. A 1.8-kb *NotI* insert of hTWEAK clone A2A, a 0.98-kb *NotI* fragment containing the hCD40L cDNA provided by Dr. E. Garber (Biogen), and a 1.46-kb *NotI* insert containing hLTα (30) were each ligated into the *NotI* site of CH269. A 0.81-kb *HindIII* insert containing the hLTβ coding region with a modified start site (30) was ligated into the *HindIII* site of CH269. 293-EBNA cells (Invitrogen) were transfected with the various CH269 vectors using LipofectAMINE, and after 2–3 days cells were either removed with Ca/Mg-free PBS with 5 mM EDTA for FACS analysis or subjected to metabolic labeling. Twice as much total DNA was used in the LTα/LTβ cotransfection experiments as in the single vector experiments.

A rabbit anti-TWEAK serum was prepared by immunizing rabbits with recombinant hTWEAK in complete Freund's adjuvant by intralymph node injection, boosting subcutaneously after 3 weeks with TWEAK in incomplete Freund's adjuvant, followed by bleeding 7 days later. This serum was functional in FACS, immunoprecipitation, and Western formats. An IgG fraction of this serum was isolated and used versus purified rabbit IgG fraction as a control. Both the FACS and immunoprecipitation procedures in addition to the rabbit anti-TWEAK utilized the following antibodies: 104c (anti-hTNF), AG9 (anti-hLTα), B9 (anti-LT), 5C8 (anti-CD40L); purified isotype-matched mAbs were used as controls (30, 31). FACS analysis was carried out in RPMI medium containing 10% FBS and 50 µg/ml heat-aggregated human IgG with the antibodies at 5 µg/ml. Phycoerythrin-labeled anti-mouse or rabbit IgG (Jackson ImmunoResearch) was used to detect antibody binding and cells were counterstained with 7-amino-actinomycin D for live gate exclusion of dead cells. For immunoprecipitation, 2 days after transfection, the cells were washed with PBS and transferred into Met/Cys-free minimal essential medium with glutamine, 10% dialyzed fetal bovine serum, and 200 µCi/ml each of Tran<sup>35</sup>S-label (ICN) and [<sup>35</sup>S]cysteine except for TNF and TWEAK, where only [<sup>35</sup>S]cysteine was provided. After 3 h of labeling, the cells were chased for an additional 3 h with cold cysteine/methionine. The supernatants and cells were harvested, lysed in 1% Nonidet P-40, and subjected to immunoprecipitation and SDS-PAGE analysis as described (30).

**Analysis of Interleukin-8 Secretion**—Cells were grown to confluence in six-well plates, at which point various cytokines were added in a total of 5 ml of medium. Aliquots were taken at various time points and assayed using a human IL-8-specific enzyme-linked immunosorbent assay that has been described previously (32).

**Cytotoxicity Assays**—Cell growth assays were carried out as described previously (33). For microscopy, HT29–14 cells were seeded into two-chamber slide well plates at a density of 100,000 cells/well and grown for 2 days. Human TWEAK, TNF, or anti-Fas (CH11, Kamaya) were added along with 80 units/ml human interferon-γ. After 24 h, the medium was removed and it was observed that slides treated with cytokine or anti-Fas antibody included many dead cells that had detached from the plastic. The remaining cells were acetone-fixed and washed into PBS containing 1 µg/ml DAPI dye. After 2 min, the dye was removed, and cells were washed into PBS and examined by fluorescence microscopy.

## RESULTS

**Isolation of Mouse and Human TWEAK cDNAs**—During the course of a study intended to clone a RNA that hybridized to an erythropoietin probe (34), we isolated several unrelated cDNAs from mouse peritoneal macrophage mRNA. One of these cDNAs was a TNF family member, as defined using the Prosite program to identify TNF sequence motifs. This new protein was thus named TWEAK in view of its TNF relatedness and

A

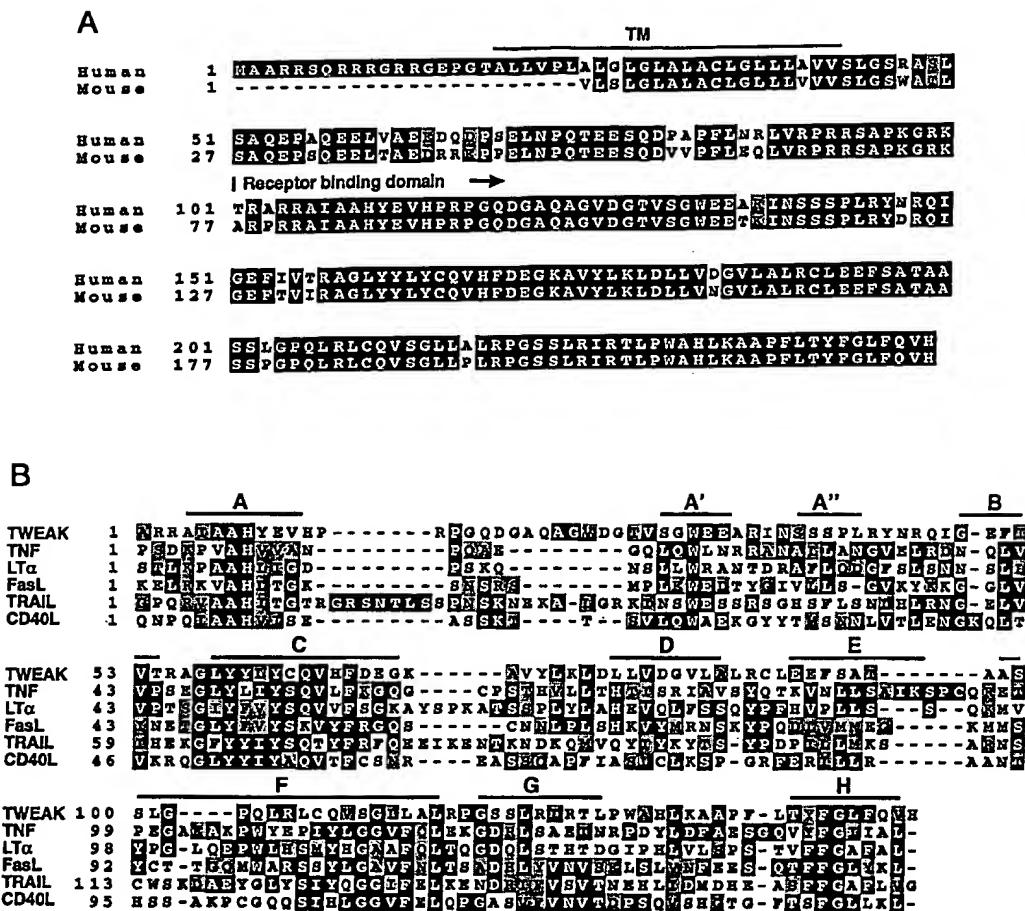
B

Human	CCCTTGAGAATTCCCTGTGGATTTTAAACAGATATTATTTTATT
Mouse	CCCTGGAA...TCCCTGTGGATTTTGAAAAGATACTATTATTTATT
Human	ATTATTTGTACAAAATGTTGATAATGGATATTAAATAGAATAAGTC
Mouse	ATTATTTGTACAAAATGT...TAAATGGATATTAAAGAGATAAATC

weak ability to induce cell death. The 1.18-kb cDNA contained the entire coding region except for an estimated 20–25 amino acids from the N terminus. A homologous human EST sequence was identified from which a PCR probe was prepared and used to screen human tonsil and fetal liver libraries. Three 1.9-kb and two 1.3-kb hTWEAK cDNAs were isolated that encoded the predicted polypeptide sequence shown in Fig. 1A. The 3'-untranslated region was identical in all six clones, and one of these clones possessed a poly(A) tail. In Fig. 1A, the two potential polyadenylation sites are indicated. In the mouse clone, polyadenylation occurred 15 bp 3' of the second region indicating utilization of this site, which is an exact AATAAA sequence in the mouse gene. In the one polyadenylated human TWEAK cDNA clone, polyadenylation occurred 14 bp from the first site, and therefore the ATTAAA is functional. Immediately upstream of the first possible polyadenylation site lies an extensive AU-rich region that resembles an adenylate/uridylate-rich element (35). These regions can destabilize the mRNA and are found in many genes including several cytokines involved in inflammatory responses. This region in TWEAK is very well conserved between mouse and man (Fig. 1B). By Northern analysis, the primary mRNA species is 1.4–1.5 kb and the long

5' region in the 1.9-kb clones is most likely an artifact. In three of the human clones, the region 5' of the transmembrane region contained an open reading frame extending for 42 amino acids beyond that shown in Fig. 1A and the initiating AUG shown here is the second AUG in this reading frame. The second AUG is preceded by a reasonable Kozak consensus sequence, which does not exist in front of the first AUG (36). The two 1.3-kb clones lacked the first AUG, leading us to suspect that the N terminus of TWEAK is that shown in Fig. 1A.

The lack of a hydrophobic leader sequence, yet the presence of a single internal hydrophobic domain of 27 amino acids in the N-terminal region, indicated that TWEAK is a type II membrane protein (Fig. 2A). All members of the TNF family of ligands appear to be type II membrane proteins, which in the cases of TNF and LT $\alpha$  are readily cleaved from the transmembrane region. The predicted short N-terminal hydrophilic 18 amino acids are very basic, a feature that is commonly observed in TNF family members and is suggestive of a stop transfer function. The transmembrane domain was linked to the receptor binding domain via a stalk region containing a high proportion of basic amino acids, suggesting that this region could be sensitive to proteolysis. The C-terminal extracellular do-



**FIG. 2. Comparison of protein sequences in the TNF ligand family.** *A*, comparison of mouse and human TWEAK amino acid sequences where identical residues are in *black* and conserved residues *shaded*. *B*, ClustalW alignment of human TWEAK to several members of the TNF family. Identical residues are in *black*, and conserved residues are *shaded*. The  $\beta$  sheet structures of LT $\alpha$  are indicated by the *bars* using the labeling defined previously (43).

main possessed a single N-terminal glycosylation site at position 139, which is a somewhat unusual location for a sugar in this structure, and this site is utilized. An amino acid sequence comparison of human and mouse TWEAK (Fig. 2A) showed 93% identity in the extracellular receptor binding domain (from amino acid 101 in Fig. 1). This high level of identity is very unusual within the family. For example, a similar comparison of TNF, TRAIL, Fas ligand, and CD40 ligand showed the mouse and human receptor binding sequences to be 79%, 75%, 86%, and 75% identical, respectively. Alignment of the human TWEAK sequence with the members of the TNF family revealed considerable structural similarity, especially in several regions previously described as characteristic of TNF family members (Fig. 2B) (5).

To determine the genomic location of hTWEAK, genomic DNA from monochromosomal somatic cell hybrids was analyzed by PCR using specific hTWEAK primers situated in the 3'-untranslated region. This analysis showed that the hTWEAK gene lies on chromosome 17 (data not shown). Further mapping using the Genebridge 4 Radiation Hybrid DNA panel showed that the gene was localized to the tip of the p13 region of chromosome 17 (LOD score 15) as shown in Fig. 3. Human TWEAK does not cluster with any of the known TNF ligands, and, while NGF is located on chromosome 17, it is on the other arm. Mouse chromosome 11 is syntenic with this region.

**TWEAK mRNA Expression in Tissues**—The 1.4–1.5-kb hTWEAK mRNA was found to be abundant in most tissues, and this pattern resembles that of TRAIL, albeit with some

tissue-specific differences (Fig. 4). Message was abundant in human heart, pancreas, colon, small intestine, lung, ovary, and prostate, while kidney, testis, and liver contained the lowest levels of TWEAK. The lymphoid organs including spleen, lymph node, appendix, and peripheral blood lymphocytes contained abundant TWEAK mRNA, whereas less was found in thymus and bone marrow. This pattern suggests that, within the immune system, the secondary immune system predominantly expresses TWEAK. In a parallel analysis of mouse tissue Northern blots, a 1.4-kb TWEAK mRNA species was also abundant in heart tissue, but there were several differences from the human pattern, *e.g.* expression was low in the mouse spleen and high in the lung. The basis for these discrepancies is not clear.

Among human cell lines, the nonlymphoid tumor cells expressed TWEAK, while the hematopoietic lineage lines HL60, K562, MOLT4, and Raji exhibited very weak or no expression (Fig. 4). Analyses of various mouse cell lines indicated that freshly isolated thioglycollate-induced peritoneal macrophages expressed a 1.4-kb TWEAK transcript and a limited survey suggested that among the hematopoietically derived T, B, and monocytic lineages, primarily monocytes express TWEAK. Additional work will be required to properly evaluate the expression pattern in primary cells.

**TWEAK Can Be Secreted**—To analyze whether TWEAK is a secreted or cell associated cytokine, we compared TWEAK to two cell-surface ligands, *i.e.* CD40 ligand (CD40L) and the heteromeric LT $\alpha$ / $\beta$  complex, and two secreted ligands, *i.e.* TNF

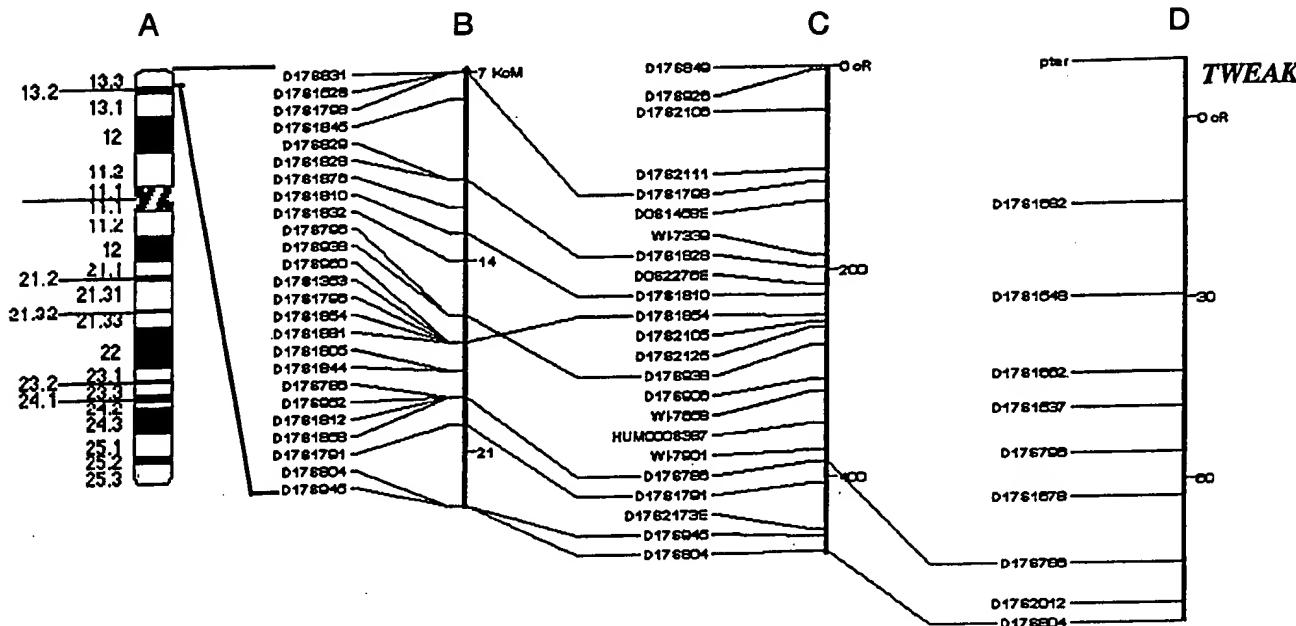


FIG. 3. Chromosomal location of human TWEAK. *A*, schematic diagram of human chromosome 17; *B*, the Genethon chromosome 17 linkage map; *C*, the Stanford human genome center map; *D*, the Whitehead DR11 radiation hybrid map. The Genebridge 4 radiation mapping panel was used to map TWEAK 38.7 cR telomeric to the framework marker D17S1682 (WI-9674) placing TWEAK distal to the genetic markers D17S786. Although radiation hybrid maps are not anchored to the cytogenetic maps, the most likely location of TWEAK is 17p13.3.

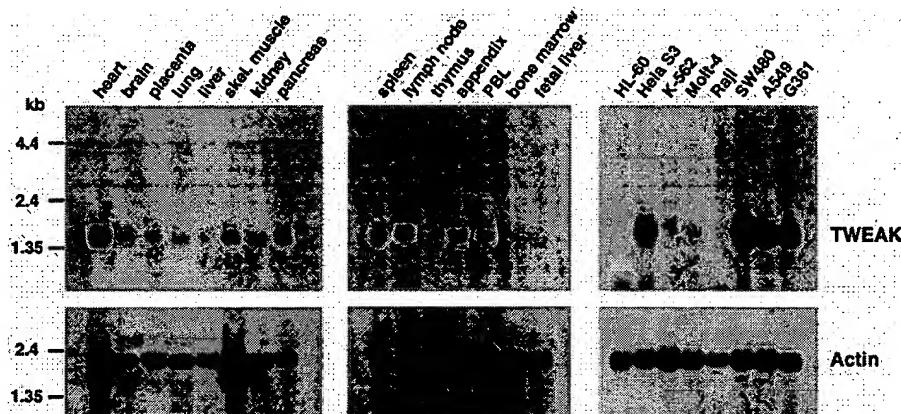
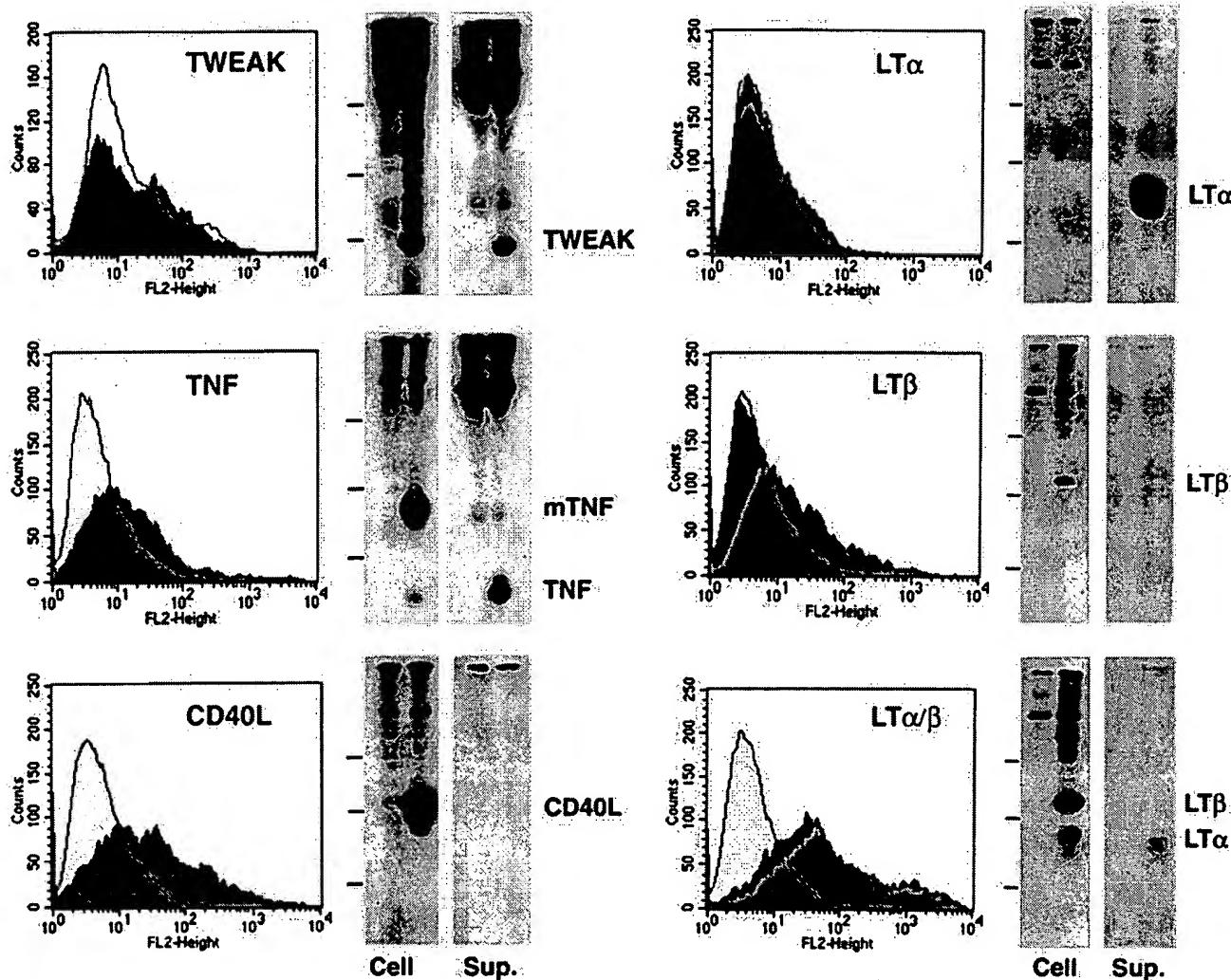


FIG. 4. Northern analysis of hTWEAK expression in various tissues. The 1.4-kb hTWEAK and actin transcripts are indicated. The cell line blot probed with hTWEAK was exposed longer than the other blots to reveal the low level signals in some of the lymphoid lines.

and LT $\alpha$ . Full-length constructs of TWEAK, TNF, CD40L, LT $\alpha$ , LT $\beta$ , and LT $\alpha$  plus LT $\beta$  were transfected into 293-EBNA human embryonic kidney cells. Vector-alone controls were negative in all analyses (data not shown). FACS analysis of the cells showed that the heteromeric LT $\alpha$ / $\beta$  complex and CD40L were retained on the cell surface at high levels (Fig. 5). TWEAK and TNF were also detectable on the cell surface, although there was variation from experiment to experiment in the levels on the cell surface. Fig. 5 shows roughly the highest levels of surface TWEAK ever observed; sometimes, no TWEAK forms were present. With TNF, sometimes higher surface levels were noted. Upon continued culturing of the transfected cells, TNF and TWEAK expression disappeared, whereas surface CD40L and LT $\alpha$ / $\beta$  complex were long-lived. Cells from the same transfection were metabolically labeled with [ $^{35}$ S]cysteine and methionine, and the secreted and cell-associated forms were examined by immunoprecipitation. An 18-kDa TWEAK form was immunoprecipitated from the 293-EBNA cell supernatants representing secreted TWEAK. Total TWEAK expression was low relative to the CD40L or LT ligands, possibly reflecting the presence of adenylate/uridylate-rich element motifs in the full-length construct. An additional complication is the presence of

only cysteine residues in the secreted TNF and TWEAK forms and the relative labeling efficiencies of methionine *versus* cysteine may give the appearance of poor expression. As expected, 17-kDa TNF and 25-kDa LT $\alpha$  were found in the supernatant and upon cotransfection with LT $\beta$ , secreted LT $\alpha$  levels appeared to decrease substantially, possibly indicating that LT $\alpha$  prefers being complexed on the cell surface. LT $\beta$  and CD40L were not readily detected in the supernatant which is consistent with their primarily cell-to-cell contact specific roles (a relatively small amount of secreted 18–20-kDa CD40L was observed with long exposures). As can be seen by the intensities of the background bands, the exposures for the TNF and TWEAK supernatants are longer than those shown for the cell lysates in Fig. 5, reflecting inefficient secretion of these ligands. PhosphorImager quantitation of the bands in this experiment showed that of the total 18-kDa TWEAK synthesized, about 5% was in the supernatant. This value was comparable to the TNF case where about 4–5% of the total TNF synthesized was cleaved to the 17-kDa form and of this amount 3–4% was secreted. Presumably, low levels of the TNF-converting enzymes and TWEAK-processing enzymes limit secretion in this cell line. In other experiments, up to 25–50% of the total la-



**FIG. 5. Comparison of the cell-associated and secreted forms of TWEAK, LTs, TNF, and CD40L from transfected 293-EBNA cells.** Each panel shows FACS analysis of human embryonic kidney cells transfected with TWEAK, TNF, CD40 ligand, LT $\alpha$  alone, LT $\beta$  alone, and LT $\alpha$  plus LT $\beta$  expression vectors. Cells were stained with specific antibodies (solid histograms) or control antibodies (open histograms) to each protein. In the LT panels, the cells were stained with anti-LT $\beta$  (gray histogram) or anti-LT $\alpha$  (black histogram). Next to the FACS profile is a SDS-PAGE analysis of the metabolically labeled immunoprecipitates from the cell lysates and the supernatants from the same cells. The first and second lanes in each panel result from the control antibody and anti-ligand antibody immunoprecipitations respectively. The positions of the 43-, 30-, and 18-kDa molecular size markers are indicated. In the TNF panel, 17-kDa secreted and 26-kDa membrane forms of TNF are indicated as TNF and mTNF, respectively. The entire immunoprecipitates from the cells and supernatants were loaded onto the gel allowing direct comparison of the relative amounts of secreted versus cell-associated material. Exposures were as follows: all LT panels and CD40L cells, 4 h; CD40L supernatant, TWEAK cells, and TNF cells, 20 h; TWEAK and TNF supernatants, 72 h.

beled TWEAK was secreted.

Immunoprecipitation of the ligands from cell lysates showed TNF to be present in its expected 26-kDa membrane form, and the LT $\alpha$  and CD40L ligands also were present at their expected 22–25- and 31–33-kDa sizes (30, 31). Cell-associated TWEAK existed in a 18-kDa processed form and in 30- and 35-kDa forms, presumably with intact transmembrane domains. A series of intermediate cleavage products were observed in this experiment. In a similar study of Fas ligand secretion from Cos cells, a low percentage of Fas ligand was cleaved, and like the TNF data presented here only a small of material was secreted (37). In contrast, even in pulse-chase experiments, it is not possible to see uncleaved LT $\alpha$  forms. TWEAK resembles the LT $\alpha$  case, since much of the cell-associated material is already cleaved away from the transmembrane region. We conclude that TWEAK, like LT $\alpha$ , is cleaved early during synthesis and transits to the outside, but, like TNF, some uncleaved molecules are retained on the cell. These observations indicate that TWEAK is primarily a secreted cytokine like TNF and LT $\alpha$ .

Further evidence for this contention was provided during the course of preparing recombinant TWEAK. A soluble molecule was prepared by deleting the N-terminal and transmembrane domains and then adding a VCAM signal sequence followed by a 11-amino acid Myc peptide tag to amino acid 67 of hTWEAK to create a secreted form basically as described for the LT $\beta$  ligand (29). Following expression in insect cells, the protein was purified from the supernatant. Two proteins were isolated, a 29-kDa full-length form wherein the VCAM leader was properly processed from the Myc-tagged N terminus and a second 17-kDa form resulting from proteolytic cleavage after Arg<sup>93</sup> (Fig. 6). The stalk region between the transmembrane domain and the  $\beta$  sheet extracellular receptor binding domain is very rich in basic amino acids in both the human and mouse proteins, and the Arg<sup>92</sup>-Arg<sup>93</sup> sequence renders the full-length protein very susceptible to proteolysis in the insect cell supernatants. This putative cleavage region is identical in the mouse TWEAK structure. The amount of TWEAK N-terminal peptide remaining in the processed form that precedes the folded  $\beta$

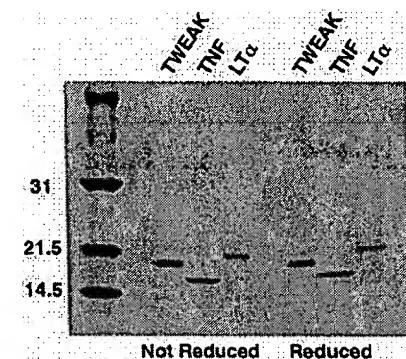


FIG. 6. Purification of secreted hTWEAK. SDS-PAGE of recombinant human TNF, LT $\alpha$ , and insect cell-derived TWEAK under reduced and nonreduced conditions.

TABLE I  
Induction of IL-8 secretion by TWEAK and other TNF family members

Activating agent	Concentration of IL-8 in the media <sup>a</sup> from cell lines:				
	Concentration	HT29	A375	WI-38	A549
ng/ml					
None		0.18	1.5	3.4	1.8
TWEAK	100 ng/ml	1.2	110	11	1.3
TWEAK boiled	100 ng/ml	0.14	4.5	3.4	1.4
TNF	50 ng/ml	15	625	275	110
TRAIL <sup>b</sup>	50 ng/ml	0.8	42	3.4	6.5
LT $\alpha$ 1/2	100 ng/ml	0.26	40	16.5	14
Anti-Fas	100 ng/ml	2.5	5.5	3.5	2.0
IFN $\gamma$	100 units/ml	nd	13	3.4	1.6

<sup>a</sup> The IL-8 concentration in the cell supernatant was measured 2 days after addition of the activating agent.

<sup>b</sup> FLAG-TRAIL was added with 1  $\mu$ g/ml M2 anti-FLAG antibody.

sheet structure resembles TNF closely. When recombinant 17-kDa TWEAK was added directly to the metabolically labeled material secreted from 293-EBNA cells, the radiolabeled form of TWEAK migrated about 1 kDa larger than the 17-kDa recombinant TWEAK in the SDS-PAGE analysis. Therefore either this same Arg-Arg site is utilized and carbohydrate differences account for the small  $M_r$  disparity or a more N-terminal residue is the site of processing in a mammalian system. Mass spectroscopic analysis of recombinant TWEAK forms indicated that a *N*-linked glycosylated form is present (data not shown). The protein is very basic and sticks to most gel exclusion chromatographic matrices under low salt conditions. Gel exclusion chromatographic analysis of TWEAK under high salt conditions was consistent with the bulk of the protein being in a trimeric form (data not shown).

**TWEAK Can Induce Chemokine Secretion**—Several of the TNF family members are known to induce chemokine secretion from various cell types (38, 39), and four cell lines were surveyed for the secretion of IL-8 in response to TNF, anti-Fas, TRAIL, LT $\alpha$ 1/2, or TWEAK treatment. Table I shows the levels of IL-8 secreted within a 2-day period from the HT29, A375, A549 and WI-38 lines, which are, respectively, a colon carcinoma, melanoma, lung carcinoma, and fibroblast lines. TNF was the best inducer of IL-8 release in all four cell lines. All cell lines except the A549 cells responded to TWEAK signaling with increased IL-8 release, and, in this capacity, TWEAK resembles LT $\alpha$ 1/2 and Trail. The A375 line exhibited the largest induction of IL-8, and here both TWEAK and LT $\alpha$ 1/2 had maximal effects in the 50–100 ng/ml range. The TWEAK effects peaked by 2 days, and boiling inactivated TWEAK's activity. Therefore, TWEAK has the potential to trigger chemokine production *in vivo* by selective target cells.

**Cytotoxic Activity of TWEAK**—Purified TWEAK induced the

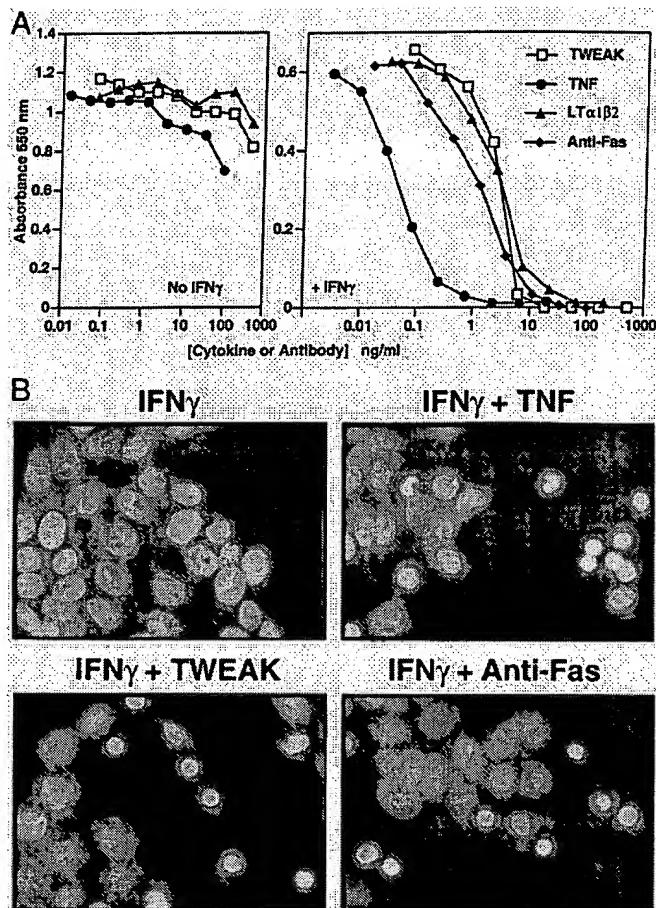


FIG. 7. TWEAK is cytotoxic to the human adenocarcinoma line HT29. A, ability of the TNF, TWEAK, LT $\alpha$ / $\beta$ , and anti-Fas (CH11) to block the growth of the HT29 line in the presence of human IFN $\gamma$ . Cells were grown for 4 days in the presence of the various agents, and growth was assessed by MTT staining. B, morphology of the cells undergoing cell death. Cells were pre-grown for 2 days and then treated for 24 h with 80 units/ml IFN $\gamma$  with either no further addition, 20 ng/ml hTNF, 100 ng/ml hTWEAK, or 100 ng/ml anti-Fas antibody CH1 in the bottom panel. DAPI staining of the nuclei of acetone-fixed cells shows nuclear condensation characteristic of apoptosis in the TNF, TWEAK, and anti-Fas-treated panels. One mitotic figure is visible in the TWEAK panel.

death of HT29 cells when cultured with human interferon- $\gamma$  (Fig. 7A). This cell is also sensitive to the activation of the TNF, LT $\beta$ , and Fas receptors as well as the TRAIL receptor.<sup>2</sup> In the presence of these agents, HT29 cells undergo apoptosis as indicated by nuclear condensation, *i.e.* small DAPI dye bright nuclei (Fig. 7B). Furthermore, we had previously shown that TNF and Fas receptor activation led to TUNEL-positive staining in these cells again indicative of an apoptotic event (26). The morphology of the dying cells was similar in the presence of an anti-Fas antibody, TNF, or TWEAK. The HT29 cells died within 16–30 h in the presence of TWEAK, which was similar to TNF- and Fas-induced cell death yet faster than LT $\alpha$ / $\beta$ -induced death. Addition of soluble TNF-R55, TNF-R75, Fas, and LT $\beta$ R immunoglobulin fusion proteins had no effect on TWEAK-induced cell death indicating that these receptors do not bind TWEAK. Preliminary surveys have shown that TWEAK does not bind to TRAMP (DR3/WSL-1/Apo-3)<sup>2</sup> or HVEM.<sup>3</sup> No other cell lines were observed to die in the presence of TWEAK, although an anti-proliferative effect was seen on

<sup>2</sup> J. Tschopp, personal communication.

<sup>3</sup> C. Ware, personal communication.

A375 cells (Table II). HeLa cells underwent a morphology change yet did not die, suggestive of a differentiation type event. Notably, hTWEAK was not cytotoxic to any lymphoid cell lines or to the mouse WEHI 164 fibroblastoid cells, which are extremely sensitive to TNF and LT. Preliminary analysis of TWEAK binding to cell surfaces suggests that a putative TWEAK receptor is present on many nonlymphoid cell lines. Thus, the simple presence of receptor does not necessarily confer on a cell the ability to induce cell death, which has been a common observation in the TNF field.

#### DISCUSSION

This paper describes the molecular cloning, expression, and biological activity of a new member of the TNF family. Both the murine and human TWEAK proteins exhibit all the characteristics of this family, *i.e.* a type II membrane protein organization and conservation of the sequence motifs involved in the folding of the protein into the TNF anti-parallel  $\beta$ -sheet structure. All members of the TNF ligand family are believed to be compact trimers, and our biochemical analysis of TWEAK is consistent with this quaternary structure. A striking feature of TWEAK is the extensive sequence conservation of the receptor binding domain between mouse and man, and only the Fas ligand approaches this level of conservation. It is enticing to speculate that this sequence conservation reflects a critical functional role for TWEAK. Within the genome, both ligands and receptors in this family are often found in clusters of genes; however, the TWEAK gene does not lie within any known cluster, nor is it in a region with known disease linkage.

TNF family members can best be described as master switches in the immune system controlling both cell survival and differentiation, although the recent description of bone density regulation by the TNF family member osteoprotegerin certainly suggests broader roles (12). There may be some clues to TWEAK's functional role from the limited characterization presented here. Only TNF and LT $\alpha$  and possibly Fas ligand are currently recognized as secreted cytokines, contrasting with the other predominantly membrane anchored members. While a membrane form of TNF has been well characterized and is

likely to have a unique biological role, secreted TNF functions as a general alarm signaling to cells more distant from the site of the triggering event. Thus, TNF secretion can amplify a primary inflammatory event leading to the well described changes in the vascular lining and consequent cell trafficking. In contrast, the membrane-bound members of the family send signals through the TNF type receptors only to cells in direct contact. For example T cells probably provide CD40-mediated "help" only to those B cells brought into contact via cognate interactions. The fact that TWEAK appears to be efficiently secreted suggests that its role will resemble that of TNF, *i.e.* to provide a long range signal. The presence of a possible AU-rich motif may indicate involvement in host defense.

TWEAK RNA is abundantly expressed in many organs in a pattern reminiscent of TRAIL (5). Other TNF family members are typically more difficult to detect in tissue Northern blots, *e.g.* CD40 ligand or TNF, where expression is limited to very specific circumstances. While TWEAK RNA is abundant, it remains to be seen if protein expression is equally abundant. TWEAK and TRAIL expression patterns suggest more constitutive functions for these TNF family members, possibly indicating that they form a subclass within the family. The relative lack of TWEAK expression in hematopoietically derived tumor lines also points to a divergence from the standard TNF family ligand, which is typically expressed in lymphoid cells.

TWEAK can induce chemokine secretion, which is likely to be a common feature of members of the TNF family. Chemokine regulation by TNF members may underlie several key aspects of their biology as is well described for TNF (38, 39), but may also extend to the LT system and BRL-1-interacting chemokines (40). The ability to induce programmed cell death is also an important and well studied feature of several members of the TNF family. Fas-mediated apoptosis appears to play a role in the regulation of autoreactive lymphocytes in the periphery and possibly the thymus (18, 23), and recent work has also implicated the TNF and CD30 systems in the survival of T cells and large cell anaplastic lymphoma lines (22, 24, 25, 41). TWEAK induced cell death in a human adenocarcinoma cell line, HT29. We and others had previously shown that the death of this line in response to TNF, Fas, or LT $\beta$  receptor signaling has the features of apoptosis (26, 39) and the death induced by TWEAK was similar to that triggered by Fas or TNF receptor activation. In contrast to the broad spectrum of Fas ligand- or TRAIL-sensitive cells, other TWEAK-sensitive cells were not readily found, and this pattern is similar to that described for LT $\beta$  receptor activation. Whether TWEAK has a function similar to Fas remains to be seen; however, the inability to kill the sensitive Jurkat and SKW 6.4 lines suggests that regulation of lymphocyte death or survival is not its role. LT $\beta$  receptor activation can induce growth arrest in some cell lines (38), and our studies on the effects of LT $\beta$  receptor activation on the growth of tumors *in vivo* are also consistent with growth arrest as opposed to direct cell death.<sup>4</sup> For these reasons, TWEAK is likely to induce cell differentiation *in vivo* and probably not cell death.

TABLE II  
Cytotoxic effects of human TWEAK on various cell lines

Cell line	Type	Cytotoxicity <sup>a</sup>
Hematopoietic		
Jurkat	T lymphoma	—
SKW 6.4	EBV B cell	—
Namalwa	Burkitt lymphoma	—
K562	Promyelocytic	—
THP-1	Monocytic leukemia	—
Nonhematopoietic		
HT29	Colon adenocarcinoma	++ <sup>b</sup>
ME-180	Cervical carcinoma	—
HeLa	Cervical carcinoma	— <sup>c</sup>
MCF-7	Breast adenocarcinoma	—
A375	Melanoma	— <sup>d</sup>
293	Embryonic kidney cells	—
WEHI 164	Mouse fibroblast	—

<sup>a</sup> 3–5 day proliferation assay in the presence and absence of human IFN $\gamma$  using a range of protein concentrations.

<sup>b</sup> Cytotoxicity was only observed in the presence of IFN $\gamma$ .

<sup>c</sup> Morphology changes.

<sup>d</sup> Some anti-proliferative effect.

<sup>4</sup> F. Mackay and J. Browning, unpublished observations.

TABLE III  
Grouping of various TNF family signaling pathways by cytotoxicity patterns

Group	Receptor
Potent inducers of apoptosis in many cell types	TNF-R55, Fas, TRAIL-RI (DR4), TRAIL-RII (DR5), TRAMP (DR3)
Weak inducers of apoptosis in a few cell types	LT $\beta$ -R, TWEAK-R, <sup>a</sup> CD30, CD27, TNF-R75
Do not induce cell death	CD40, OX-40, 4-1BB

<sup>a</sup> TWEAK-R is presumed to exist.

It is possible to segregate the TNF receptor pairs into three groups based on their ability to induce cell death (Table III). First, TNF-R55, Fas, TRAIL-RI (DR4), TRAIL-RII (DR5), and TRAMP (DR3/WSL-1/Apo-3) receptors can efficiently induce cell death in many lines, and these receptors have canonical death domains (8–10, 23). Next, there are those receptors that trigger a weaker death signal limited to a few cell types; the TWEAK, CD30, LT $\beta$ , and possibly the TNF-R75 (42, 43) and CD27 receptors (44) are examples of this class. Finally, there are those members that cannot efficiently deliver a death signal, and the possibility must be considered that these receptors have simply not been studied as well. Probably all groups can exhibit antiproliferative effects on some cell types consequent to inducing cell differentiation, e.g. CD40 (45).

How the “weak death” group can trigger cell death in the absence of a canonical death domain is an interesting question and prompts speculation that an alternative entry point into the death-inducing caspase cascade exists. Specifically, in the case of HT29 cells, why does receptor signaling lead to death only in the presence of IFN $\gamma$ ? It is possible that these weak death receptors may activate the conventional death domain-mediated pathways via induction of the expression of Fas and Fas ligand or other receptor/ligands in the strong death group. In support of this concept, Fas expression is known to be up-regulated by IFN $\gamma$  on HT29 cells, and the expression of Fas ligand by tumor lines as a possible immune surveillance escape mechanism has been described (46–48), although the anti-hFas-L blocking antibody, NOK-1, did not affect cell death in this system (data not shown). We have also observed that activation of the LT $\beta$  receptor can potentiate TNF signaling possibly reflecting favorable cross-talk between signaling pathways (26, 49). Alternatively, weak death receptor signaling may differentiate the line into a state of unfulfilled growth factor dependence or cell cycle confusion with consequent initiation of the death pathway. A fourth possibility lies in the recent observations on the ability of NF- $\kappa$ B activation to inhibit the death signal delivered by death domain bearing receptors and IFN $\gamma$  treatment may relieve the death repression by NF- $\kappa$ B (23). Since anti-Fas-induced HT29 cell death is IFN $\gamma$ -dependent yet Fas signaling does not lead to NF- $\kappa$ B activation (23), regulation of downstream NF- $\kappa$ B-mediated events is less likely. Finally, and most likely, death may be initiated by other “non-FLICE”-initiated signaling pathways such as the ceramide or JNK/stress-activated protein kinase pathways (50, 51). Whatever the mechanism, further exploration of the death trigger initiated by these weak death receptors may provide an additional approach to cancer therapy.

The TNF family has grown dramatically in recent years to encompass at least 14 different ligand/receptor signaling pathways that regulate host defense and the immune system. The widespread expression patterns of TWEAK and TRAIL indicate that there may be considerably more functional variety to be uncovered in this family. This aspect was highlighted recently with the discovery of two new TNF receptors that affect the ability of Rous sarcoma and herpes simplex virus to productively infect cells along with the discovery of a bone density regulating receptor. When coupled with the historical observations that TNF itself has anti-viral activity and that pox viruses encode for decoy TNF receptors to avoid host defense, it appears that viral pathology and the functions of the TNF are interwoven (6, 7, 13, 52). The generation of soluble TWEAK and the eventual identification of the TWEAK receptor should provide the tools to elucidate the biological function of this new pathway.

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